

Preliminary communication

GROWTH AND HAEMOLYTIC ACTIVITY OF PATHOGENIC *VIBRIO* SPECIES IN EGG-FRIED-RICE WITH DIFFERENT EGG RATIOS

R.D. WANG^a, Y.J. DENG^a, L.J. SUN^{a*}, Y.L. WANG^{a*}, Z.J. FANG^a, D.F. SUN^a, Q. DENG^a
and R. GOONERATNE^b

^aCollege of Food Science and Technology, Guangdong Ocean University, Guangdong Provincial Key Laboratory of Aquatic Product Processing and Safety, Key Laboratory of Advanced Processing of Aquatic Products of Guangdong Higher Education Institution 524088, China

^bDepartment of Wine, Food and Molecular Biosciences, Faculty of Agriculture and Life Sciences, Lincoln University, Lincoln 7647, Canterbury, New Zealand

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Growth and haemolytic activity of several pathogenic *Vibrio* species were compared in egg-fried-rice with different egg ratios. Egg-fried-rice preparations with rice-to-egg ratios of 4:1, 1:1, and 1:4 were inoculated with either *Vibrio parahaemolyticus*, *V. cholerae*, *V. vulnificus*, or *V. alginolyticus* and incubated for 24 h. Cell number, thermostable direct haemolysin (TDH) activity, and total haemolytic activity were determined. The cell number and total haemolytic activity increased in all *Vibrio* strains after 24 h, and these were most marked in egg-fried-rice with the highest egg content (1:4 (rice:egg) ratio; $P < 0.05$). *V. alginolyticus* exhibited the maximal growth and *V. parahaemolyticus* the highest haemolytic activity, but only *V. parahaemolyticus* ATCC 33847, *V. alginolyticus* CAMT 21162, and *V. alginolyticus* HY 91101 showed TDH activity. Results suggest that lowering egg content in egg-fried-rice could reduce growth and virulence of *Vibrio* pathogens.

Keywords: egg-fried-rice, growth, pathogenic *Vibrio* species, thermostable direct haemolysin activity, total haemolytic activity

Vibrio species are Gram-negative bacteria that occur in coastal waters and maintain ecological balance in the marine environment. Of all *Vibrio* species, *V. cholerae* (*Vc*), *V. parahaemolyticus* (*Vp*), *V. alginolyticus* (*Va*), and *V. vulnificus* (*Vv*) are human pathogens (BOWERS & DEPAOLA, 2010). Consumption of seafood contaminated with pathogenic *Vibrio* spp. may cause acute gastroenteritis characterized by diarrhoea, vomiting, abdominal cramps, and systemic infections (JANDA et al., 1988). Some *Vibrio* spp. (e.g. *Vp*) can contaminate seafood and also non-seafood such as poultry, pork, eggs, and associated food such as egg-fried-rice by cross contamination and/or via cooking utensils (TUNUNG et al., 2010; WU et al., 2014; XIE et al., 2016). However, little is known about the pathogenicity of *Vibrio* spp. in non-seafood.

All pathogenic *Vibrio* spp. produce haemolysins, with thermostable direct haemolysin (TDH) regarded as the most important virulence factor (HONDA & IIDA, 1993). WANG and co-workers (2016, 2018) compared the pathogenicity of *Vp* in food matrices, and found that growth rate and haemolytic activity were significantly higher in egg-fried-rice, indicating a possible higher risk of *Vp* occurrence in this food. It is not known, however, whether the

* To whom correspondence should be addressed.

Phone: + 86 137 6309 4052; e-mails: suncamt@126.com (L.S.), wangylchina@163.com (Y.W.).

growth and haemolytic activity of *Vp* changes with the proportion of egg in egg-fried-rice. Neither is it known whether growth and haemolytic activity are different for other pathogenic *Vibrio* spp. in egg-fried-rice.

The objectives of this study are to quantify the growth, haemolytic activity, and pathogenicity of 12 strains of four *Vibrio* spp., namely, *Vp*, *Vc*, *Va* and *Vv*, in egg-fried-rice with different egg ratios.

1. Materials and methods

1.1. Bacterial strains and growth conditions

V. parahaemolyticus strains ATCC 17802 and ATCC 33847 were obtained from the China Committee for Culture Collection of Microorganisms. *Vp* (ZJS-Vp-3), *Vc* (VC 13130, VC 13131 and VC 13132), *Va* (VA 12021, CAMT 21162 and HY 91101), and *Vv* (VV 13009, VV 13010, and VV 13011) were isolated from diseased shrimp, fish, and mussel. All strains were verified (using PCR reactions for amplification of a hypervariable region of the 16S rRNA gene) and stored in 25% glycerol at -20°C . Each strain was grown in brain heart infusion broth (BLBT, Beijing, China) containing 3% NaCl at 37°C for 24 h, and the inoculum was three times passaged in the broth. The final concentrations of inoculants were adjusted to $\sim 10^5$ colony-forming units (CFU) ml^{-1} for inoculation of egg-fried-rice matrices.

1.2. Egg-fried-rice preparation and inoculation

The egg-fried-rice was prepared in the laboratory according to WANG and co-workers (2016). Briefly, egg-fried-rice with different ratios of rice to egg (wt/wt = 4:1, 1:1, 1:4) were cooked at 80°C for 20 min. Test portions (100 g, $n=3$) of egg-fried-rice, with salt added at 3% in sterile Erlenmeyer flasks, were sterilized by autoclave (YXQ-L-50A, Shanghai Boxun, Shanghai, China) at 100°C for 20 min to kill native bacteria, then transferred to a biosafety hood, cooled to room temperature, inoculated with 1 ml of pathogenic *Vibrio* spp., and vortexed (XW-80A, Qilinbei, Haimen, China) for 10 min. A 10 g sample of each inoculated test portion was analysed by the plate count method (JONGENBURGER et al., 2010) to obtain an initial bacterial count. The bacteria were serially diluted and grown in thiosulphate citrate bile salts sucrose agar medium to form individual colonies, which were quantified (colony-forming units, CFUs). The remainder of the inoculated samples were incubated at 37°C for 24 h.

After incubation, the inoculated food samples (90 g) were divided into two parts. One part (10 g) was used for bacterial quantification (as described above), and the other (80 g) was washed with 80 ml of 0.01 M phosphate-buffered saline (PBS, pH 7.2) and centrifuged (Thermo Lynx 6000, Thermo Scientific, Waltham, MA) at 12 000 r.p.m. for 20 min at 4°C . The supernatant was filtered (0.22 μm , Millipore, Billerica, MA) and stored at -20°C until used to test haemolytic activity.

The same procedure was used for control samples of the different egg ratios of egg-fried-rice except not inoculated with the pathogenic *Vibrio* spp.

1.3. Haemolysin production

1.3.1. TDH activity. TDH activity was determined as described by WAGATSUMA (1968), with slight modification. The test plate consisted of 5% rabbit red blood cells (RBCs) in Wagatsuma

agar medium (BLBT). Once the blood agar solidified in the plate, wells were made in the agar. Then, 50 µl of filtrate was added to each well and cultured for 12 h at 37 °C. The diameter of the lytic halo around each well was considered a positive reaction for detecting the extent of TDH activity.

1.3.2. Total haemolytic activity. Total haemolytic activity of egg-fried-rice filtrates was measured by the relative haemolytic activity assay (GARCIA-ARRIBAS & KRAMER, 1990). Briefly, rabbit haemocytes were obtained by centrifugation of blood (3500 r.p.m., 4 °C, 10 min), washed three times with PBS, and diluted to 5% with PBS. Subsequently, a sample (400 µl) of filtrates was mixed with 100 µl of 5% rabbit RBC in 1.5-ml sterile tubes and incubated at 37 °C for 1.5 h. Unlysed RBCs were allowed to settle to the bottom of the tube overnight at 4 °C, and 200-µl portions of the supernatant were transferred to 96-well flat-bottomed microplates (Nunc, Thermo Scientific, Waltham, MA), and the absorbance was measured at 570 nm in a microplate reader (Varioskan Flash, Thermo Scientific). The same procedure was employed for controls, except for the samples consisting of only egg-fried-rice filtrates without the pathogenic *Vibrio* spp. inoculum. The results are reported as:

$$A_{\text{relative haemolytic activity}} = A_{\text{sample}} - A_{\text{control}}$$

1.4. Statistical analysis

All data were analysed using SPSS 19.0 software (SPSS Inc., Chicago, IL, USA). Differences between the means were tested by one-way ANOVA, with the level of significance set at $P < 0.05$.

2. Results and discussion

2.1. Growth of pathogenic *Vibrio* spp. in egg-fried-rice

Egg-fried-rice can be contaminated by many microbes including *Salmonella* and *Listeria* spp. Invasive infections often develop after consumption of aquatic food contaminated by *Vibrio* spp., but there are no reports of the pathogenicity of *Vibrio* spp. in non-aquatic food. In this study, we showed that pathogenic *Vibrio* spp. can grow in egg-fried-rice with different egg ratios ($P < 0.05$, Table 1). Among the pathogenic *Vibrio* spp., *Va* exhibited the highest growth. It is now clear that egg-fried-rice can provide the nutrients required for *Vibrio* spp. growth and that infected egg-fried-rice may also account for some *Vibrio* spp. outbreaks. This finding is in agreement with reports of non-seafood-borne illnesses caused by *Vp.* through cross-contamination of cooked foods (SCIENTIFIC COMMITTEE ON ENTERIC INFECTIONS AND FOODBORNE DISEASES, 2011). Thus, *Vibrio* spp. growth in egg-fried-rice can be prevented by avoiding cross contamination of ready-to-eat foods with uncooked food or food utensils and by stepping up ready-to-eat food surveillance/monitoring.

Significantly higher ($P < 0.05$) growth of pathogenic *Vibrio* spp. were observed in egg-fried-rice with a high egg:rice ratio (4:1) compared with a low egg:rice ratio (1:4). CHIANG and CHOU (2008) indicated that the growth and toxin production of *Vp* can be augmented by increasing the nitrogen source (protein) in the medium. Protein is one of the main nutrients in eggs. So, when the egg ratio in fried rice is increased, the protein content is also increased. We hypothesize that the high protein content in egg-fried-rice promoted the growth of *Vp.* Other pathogenic *Vibrio* species (*Vc*, *Vv*, and *Va*) behaved similarly, with higher growth rate in fried rice with a higher egg:rice ratio. It appears that all pathogenic *Vibrio* spp. may have a similar demand of protein for growth.

Table 1. Growth of 12 pathogenic strains of *Vibrio* species in egg-fried-rice with different egg ratios*

Rice:egg (w/w)	<i>Vibrio</i> spp.	Strains	Cell number in egg-fried-rice (lg CFU·g ⁻¹) ± SD		
			Initial	Final	Growth [†]
4:1	<i>Vibrio parahaemolyticus</i>	ATCC 17802	2.92±0.31	7.21±0.41	4.29±0.51 ^b
		ATCC 33847	2.83±0.22	7.32±0.23	4.49±0.36 ^b
		ZJS-Vp-3	2.36±0.24	7.18±0.33	4.82±0.34 ^c
	<i>Vibrio cholerae</i>	VC 13130	2.85±0.20	7.41±0.33	4.56±0.42 ^c
		VC 13131	2.82±0.19	7.23±0.28	4.41±0.22 ^c
		VC 13132	2.79±0.22	7.30±0.30	4.51±0.26 ^b
	<i>Vibrio alginolyticus</i>	VA 12021	2.48±0.19	7.53±0.25	5.05±0.24 ^c
		CAMT 21162	2.77±0.26	7.79±0.30	5.02±0.33 ^c
		HY 91106	2.21±0.45	7.45±0.16	5.24±0.39 ^c
	<i>Vibrio vulnificus</i>	VV 13009	2.99±0.22	7.42±0.12	4.43±0.28 ^c
		VV 13010	2.44±0.50	7.38±0.22	4.94±0.13 ^c
		VV 13011	2.56±0.48	7.44±0.36	4.88±0.32 ^b
1:1	<i>Vibrio parahaemolyticus</i>	ATCC 17802	2.81±0.12	8.21±0.16	5.40±0.33 ^a
		ATCC 33847	2.92±0.26	8.30±0.20	5.38±0.36 ^a
		ZJS-Vp-3	2.88±0.26	8.20±0.10	5.32±0.22 ^b
	<i>Vibrio cholerae</i>	VC 13130	2.77±0.35	8.28±0.17	5.51±0.31 ^a
		VC 13131	2.84±0.23	8.33±0.20	5.49±0.34 ^a
		VC 13132	2.67±0.31	8.41±0.23	5.74±0.27 ^a
	<i>Vibrio alginolyticus</i>	VA 12021	2.69±0.17	8.81±0.12	6.12±0.33 ^b
		CAMT 21162	2.94±0.19	8.73±0.23	5.79±0.39 ^b
		HY 91106	2.51±0.26	8.80±0.19	6.29±0.27 ^a
	<i>Vibrio vulnificus</i>	VV 13009	2.90±0.21	8.23±0.10	5.33±0.30 ^b
		VV 13010	2.79±0.32	8.30±0.13	5.51±0.35 ^b
		VV 13011	2.63±0.16	8.11±0.11	5.48±0.28 ^a
1:4	<i>Vibrio parahaemolyticus</i>	ATCC 17802	2.61±0.38	8.41±0.26	5.80±0.40 ^a
		ATCC 33847	2.98±0.40	8.39±0.19	5.41±0.32 ^a
		ZJS-Vp-3	2.58±0.28	8.39±0.16	5.81±0.16 ^a
	<i>Vibrio cholerae</i>	VC 13130	2.84±0.30	8.09±0.12	5.25±0.24 ^b
		VC 13131	2.65±0.11	8.47±0.22	5.82±0.31 ^a
		VC 13132	2.82±0.25	8.51±0.40	5.69±0.28 ^a
	<i>Vibrio alginolyticus</i>	VA 12021	2.89±0.19	8.93±0.31	6.04±0.26 ^a
		CAMT 21162	2.92±0.22	9.00±0.33	6.08±0.28 ^a
		HY 91106	2.81±0.24	9.07±0.25	6.26±0.30 ^a
	<i>Vibrio vulnificus</i>	VV 13009	2.66±0.14	8.33±0.12	5.67±0.26 ^a
		VV 13010	2.94±0.23	8.40±0.34	5.46±0.29 ^a
		VV 13011	2.71±0.30	8.27±0.21	5.56±0.31 ^a

Note: Same-strain growth means where the three egg:rice ratios in the same strain have different superscript letters growth was significantly different (P<0.05).

* Mean ± standard deviation of three replicates.

[†]Growth = Final – Initial (log CFU·g⁻¹)

2.2. Haemolysin production of pathogenic *Vibrio* spp. in egg-fried-rice

Haemolysins control haemolytic activity, cytotoxicity, and enterotoxicity. TDH activity determines pathogenicity of *Vibrio* spp. (NISHIBUCHI & KAPER, 1995). In the TDH activity test, only the strains *Vp* ATCC 33847, *Va* CAMT 21162, and *Va* HY 91101 showed β -haemolysis of rabbit RBC on blood agar (Table 2), indicating that not all pathogenic *Vibrio* spp. can produce TDH. As TDH is encoded by *tdh* genes, we presumed that the *Va* CAMT 21162 and *Va* HY 91101 strains may also be harbouring *tdh* genes like the standard strain of *Vp* ATCC 33847. This result is consistent with findings of TANIGUCHI (1985) and THEETHAKAEW (2013) and their co-workers, who reported that very few isolated *Vibrio* spp. strains have TDH activity and only the *Vibrio* spp. strains isolated from clinical cases show TDH activity. The haemolytic zone of strains *Vp* ATCC 33847, *Va* CAMT 21162, and *Va* HY 91101 in our study significantly increased ($P < 0.05$, Table 2) in high egg ratio fried-rice (rice:egg=1:4) filtrates of *Vibrio* spp. than in the low (rice:egg=4:1) and equal ratio (rice:egg=1:1) filtrates. We hypothesize that the foods with high egg content may stimulate the *Vibrio* spp. strains that harbour the *tdh* genes to produce more TDH haemolysin and thereby increase the risk of food poisoning in all food types with a high egg content.

Total haemolytic activity reflects the degree of harm caused by *Vibrio* spp. virulence factors. All *Vibrio* spp. tested exhibited haemolytic activity (Table 2) with *Vp* > *Va*. No significant haemolytic activity was detected among *Vc* and *Vv*. We believe that people consuming *Vp*-contaminated egg-fried-rice will be affected more than those exposed to other *Vibrio* spp.

When the different egg ratios were compared, the highest haemolytic activity was observed in egg-fried-rice with the highest egg ratio (rice:egg=1:4). TANIGUCHI (1986) and SHINODA (1991) and their co-workers showed that production of lecithin dependent haemolysin, which causes haemolysis, increased at a higher lecithin concentration in the medium. IUCHI & TANAKA (1980) reported that virulence factors of *Vibrio* spp. were repressed by the various carbohydrates present in the medium. We believe that the high concentration of carbohydrates in low-egg-ratio (rice:egg=4:1) egg-fried-rice may have suppressed the *Vibrio* species' ability to secrete exoenzymes with haemolytic activity. At higher egg ratios in fried-rice, the lecithin concentration was increased, and it may have stimulated the pathogenic *Vibrio* spp. to produce more lecithin-dependent haemolysin and hence exhibit greater haemolytic activity.

3. Conclusions

Pathogenic *Vibrio* spp. exhibited increased growth and total haemolytic activity in egg-fried-rice. The highest growth was observed in *Va* strains and the highest haemolytic activity in *Vp* strains. The growth and haemolytic activity of pathogenic *Vibrio* spp. were influenced by the egg ratio in egg-fried-rice. Low egg ratios (high rice content) could limit growth and virulence factor production. High egg ratios not only significantly stimulated pathogenic *Vibrio* spp. to grow but also increased the TDH activity and total haemolytic activity.

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Table 2. Haemolytic circle diameter and total haemolytic activity of 12 pathogenic strains of *Vibrio* species in egg-fried-rice with different egg ratios*

Rice:egg (w/w)	<i>Vibrio</i> spp.	Strains	Haemolytic zone diameter (mm) \pm SD	Total haemolytic activity (OD ₅₇₀) \pm SD
4:1	<i>Vibrio parahaemolyticus</i>	ATCC 17802	–	0.93 \pm 0.04 ^c
		ATCC 33847	11.88 \pm 3.03 ^b	0.91 \pm 0.06 ^c
		ZJS-Vp-3	–	0.88 \pm 0.13 ^c
	<i>Vibrio cholerae</i>	VC 13130	–	0.48 \pm 0.16 ^c
		VC 13131	–	0.46 \pm 0.09 ^c
		VC 13132	–	0.47 \pm 0.14 ^c
	<i>Vibrio alginolyticus</i>	VA 12021	–	0.60 \pm 0.03 ^c
		CAMT 21162	7.01 \pm 1.89 ^c	0.62 \pm 0.08 ^c
		HY 91106	6.21 \pm 2.45 ^c	0.63 \pm 0.04 ^c
	<i>Vibrio vulnificus</i>	VV 13009	–	0.52 \pm 0.05 ^c
		VV 13010	–	0.51 \pm 0.10 ^c
		VV 13011	–	0.54 \pm 0.12 ^c
1:1	<i>Vibrio parahaemolyticus</i>	ATCC 17802	–	1.11 \pm 0.03 ^b
		ATCC 33847	12.30 \pm 2.22 ^b	1.20 \pm 0.07 ^b
		ZJS-Vp-3	–	1.15 \pm 0.09 ^b
	<i>Vibrio cholerae</i>	VC 13130	–	0.62 \pm 0.11 ^b
		VC 13131	–	0.65 \pm 0.10 ^b
		VC 13132	–	0.69 \pm 0.06 ^b
	<i>Vibrio alginolyticus</i>	VA 12021	–	0.82 \pm 0.04 ^b
		CAMT 21162	7.56 \pm 1.11 ^b	0.83 \pm 0.04 ^b
		HY 91106	7.62 \pm 2.32 ^b	0.86 \pm 0.03 ^b
	<i>Vibrio vulnificus</i>	VV 13009	–	0.63 \pm 0.06 ^b
		VV 13010	–	0.70 \pm 0.12 ^b
		VV 13011	–	0.65 \pm 0.06 ^b
1:4	<i>Vibrio parahaemolyticus</i>	ATCC 17802	–	1.34 \pm 0.06 ^a
		ATCC 33847	14.16 \pm 1.10 ^a	1.39 \pm 0.09 ^a
		ZJS-Vp-3	–	1.30 \pm 0.03 ^a
	<i>Vibrio cholerae</i>	VC 13130	–	0.83 \pm 0.05 ^a
		VC 13131	–	0.87 \pm 0.08 ^a
		VC 13132	–	0.84 \pm 0.06 ^a
	<i>Vibrio alginolyticus</i>	VA 12021	–	0.99 \pm 0.03 ^a
		CAMT 21162	8.13 \pm 1.24 ^a	1.03 \pm 0.08 ^a
		HY 91106	8.00 \pm 2.34 ^a	1.07 \pm 0.10 ^a
	<i>Vibrio vulnificus</i>	VV 13009	–	0.82 \pm 0.07 ^a
		VV 13010	–	0.88 \pm 0.05 ^a
		VV 13011	–	0.77 \pm 0.06 ^a

Note: Same-strain mean circle diameters for the three egg:rice ratios with different superscript letters are significantly different ($P < 0.05$).

* Mean \pm standard deviation of three replicates. In control group (without *Vibrio* inoculation), there was no haemolytic zone and no haemolysis.

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